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Efficient Self-Assembly of Human Papillomavirus Type 16 L1 and L1-L2 into Virus-Like Particles

REINHARD KIRNBAUER,[1] JANET TAUB,[1] HEATHER GREENSTONE,[1] RICHARD RODEN,[1] MATTHIAS DÜRST,[2] LUTZ GISSMANN,[2] DOUG R. LOWY,[1] AND JOHN T. SCHILLER[1]*

Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, Maryland 20892,[1] and Deutsches Krebsforschungszentrum, 6900 Heidelberg, Germany[2]

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The L1 genes of two human papillomavirus type 16 (HPV16) isolates derived from condylomata acuminata were used to express the L1 major capsid protein in insect cells via recombinant baculoviruses. Both L1 major capsid proteins self-assembled into virus-like particles (VLP) with high efficiency and could be purified in preparative amounts on density gradients. The yield of VLP was 3 orders of magnitude higher than what has been obtained previously, using L1 derived from the prototype HPV16. DNA sequence comparison identified a single nonconserved amino acid change to be responsible for the inefficient self-assembly of the prototype L1. VLP were also obtained by expressing L1 of HPV6, HPV11, and cottontail rabbit papillomavirus, indicating that L1 from a variety of papillomaviruses has the intrinsic capacity to self-assemble into VLP. Coexpression of HPV16 L1 plus L2 by using a baculovirus double-expression vector also resulted in efficient self-assembly of VLP, and the average particle yield increased about fourfold in comparison to when L1 only was expressed. Coimmunoprecipitation of L1 and L2 and cosedimentation of the two proteins in a sucrose gradient demonstrated that L2 was incorporated into the particles. The ability to generate preparative amounts of HPV16 L1 and L1-L2 VLP may have implications for the development of a serological assay to detect anti-HPV16 virion immune responses to conformational epitopes and for immunoprophylaxis against HPV16 infection.

Genital infection with human papillomaviruses (HPV) is a common sexually transmitted disease usually consisting of benign epithelial proliferation. However, infection with certain HPV types, most commonly HPV type 16 (HPV16), is also associated with progression to high-grade dysplasia and anogenital carcinoma. Infection with these high-risk HPV types is now considered to be the most significant risk factor in the development of cervical cancer, the second most common cancer of women worldwide (17, 22).

Despite recent advances in the propagation of HPV in mouse xenografts and in raft cultures (3, 12, 18), these systems are not well suited to mutational studies of HPV structural proteins and do not yield preparative amounts of HPV virions. The conformational dependency of neutralizing epitopes, as observed in experimental animal papillomavirus (PV) systems (1, 10), suggests that properly assembled HPV particles might be critical for the induction and detection of clinically relevant immune reactivity.

We and others have recently shown that expression of PV L1 major capsid proteins in eukaryotic cells can result in the self-assembly of virus-like particles (VLP) which are morphologically similar to native virions (7, 10, 16). However, for HPV16, although expression of L1 alone or coexpression of L1 along with the minor capsid protein L2 did result in VLP formation, either the VLP were significantly smaller than native virions (21) or their efficiency of self-assembly was low (10).

The relative inefficiency with which HPV16 self-assembled into VLP might be an intrinsic characteristic of high-risk mucosal HPV types, especially in view of the finding that, in contrast to lesions induced by nongenital HPVs, virions are much more difficult to detect in naturally occurring lesions associated with HPV16 or other high-risk HPV types. Alternatively, the HPV16 L1 gene which has been used in previous studies might contain one or more mutations that inhibit self-assembly. In those previous studies, the L1 gene was derived from the prototype HPV16 DNA, which was isolated from a cervical carcinoma. One theoretical argument in favor of the possibility that L1 from the prototype strain might contain a mutation is that there is probably no selective pressure for maintenance of an intact L1 gene in carcinomas, since malignant tumors are usually nonproductive with respect to virions. Condylomata acuminata, on the other hand, produce infectious virus and are therefore likely to contain HPV genomes that encode functional L1 proteins.

To determine whether inefficient self-assembly of HPV16 VLP resulted from a functional alteration in L1 of the prototype HPV16 genome or was a property intrinsic to this HPV type, we have analyzed the self-assembly of the L1 protein encoded by two different HPV16 DNAs isolated from condylomata acuminata. Using a baculovirus expression system, both clones encode L1 proteins that efficiently self-assemble into VLP. Sequence analysis of L1 indicates that one particular amino acid residue common to both of these L1 proteins is different in the prototype HPV16 L1.

MATERIALS AND METHODS

Generation of L1 and L2 recombinant baculoviruses. We constructed single-gene baculovirus transfer vectors which contain the L1 coding region (nucleotides [nt] 5637 to 7154) of two HPV16 DNA clones isolated by two of us (M.D. and L.G.) from condylomata acuminata, 114/K and 114/B, by using the cloning strategy previously described for L1 from the prototype HPV16 clone (10). These constructs were made by cloning each L1 gene into the expression vector pEV mod, resulting in L1 being under the control of the polyhedrin promoter. The L2

* Corresponding author.

open reading frame (ORF) (nt 4235 to 5656) of the prototype was also placed in this vector. The L1 genes of HPV6b (2), HPV11 (6), and cottontail rabbit PV (CRPV) (13) were cloned as *Bgl*II fragments by using the same strategy, except that the polymerase chain reaction (PCR) primers contained L1 sequences specific for these viruses. In addition, HPV16 L1-L2 double expression vectors were constructed by using pSynwtVI$^-$, such that the prototype HPV16 L2 was expressed under the control of a synthetic promoter, pSyn, and the HPV16 L1s were expressed under the control of the polyhedrin promoter. Both vectors were kindly provided by L. Miller (19). The L1 genes were cloned as 5′ *Bgl*II-to-3′ *Bgl*II fragments into pEVmod or pSynwtVI$^-$. For cloning L2 into pSynwtVI$^-$, the L2 primer sequences were as follows: sense, 5′-GCGGTGATATCAATATGCGACACAAACGTTCTGC AAAACGCACAAAACGT-3′, and antisense, 5′-CCGCTCC GCGGACTGGGACAGGAGGCAAGTAGACAGTGGCCT CA-3′. Restriction sites (underlined) were included in the oligonucleotide primers and used for cloning L2 as a 5′ *Eco*RV-to-3′ *Sst*II fragment into the baculovirus double-expression vector. For cloning L2 into pEVmod, comparable primers with *Bgl*II restriction sites at both ends were used, so that L2 was cloned as a 5′ *Bgl*II-to-3′ *Bgl*II fragment. Sequencing was carried out for the vector-HPV junctions and, for HPV16, the entire L1 and L2 ORFs of the baculovirus vectors. Numbering refers to the prototype HPV16 sequence, which is not corrected for the missing T at nt 3903 in the E5 ORF (8) and for the sequencing errors in the L1 ORF (14). Recombinant baculovirus stocks were generated by cotransfection with baculovirus DNA (Baculo-Gold; PharMingen, San Diego, Calif.), by using lipofectin (GIBCO/BRL, Gaithersburg, Md.), and plaque purification by standard techniques (10).

Generation of the HPV16 L2 rabbit antiserum. Sequences encoding a glutathione *S*-transferase (GST)-HPV16 L2 fusion protein were constructed by PCR amplification of the entire L2 ORF of the prototype, with the same primers described above, and insertion of the resulting *Bgl*II fragment into the *Bam*HI cloning site of the vector pGEX-2T (Pharmacia, Milwaukee, Wis.). The fusion protein was expressed in bacteria according to the specifications of the manufacturer, purified by preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and used to immunize a rabbit as described previously (10).

Characterization of proteins expressed in insect cells. Sf-9 cells were mock infected or infected at a multiplicity of infection (MOI) of ~10 with either wild-type or recombinant baculovirus. After 72 h, cells were lysed by being boiled in SDS sample buffer and analyzed by SDS-PAGE in 10% gels. Proteins were stained with 0.25% Coomassie blue or analyzed by Western blotting (immunoblotting) with the monoclonal antibody Camvir-1 (anti-HPV16 L1; PharMingen) or the GST-16L2 antiserum and then ^{125}I-labeled anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG, respectively.

Preparative purification of particles. For production of VLP, Sf-9 cells were grown at 27°C as 500-ml suspension cultures in 1-liter spinner flasks (Bellco, Vineland, N.J.) by using Grace's supplemented insect medium with 10% fetal calf serum, penicillin-streptomycin, and 0.1% pluronic F-68 (all from GIBCO/BRL). One liter of cells at a density of 3×10^6 to 3.5×10^6/ml was harvested by low-speed centrifugation ($200 \times g$) and infected at a MOI of ~10 in 100 ml of Grace's medium without fetal calf serum for 1 h with periodical inversion. The cells were then grown as adherent cultures by plating them into 20 tissue culture plates (245 by 245 mm; Nunc, Naperville, Ill.) in a volume of 90 ml of Grace's medium per plate, with 10% fetal calf serum. After 72 h, the cells were harvested by scraping and centrifuged at $1,100 \times g$ (Sorvall RC-3B) for 5 min and washed once with ice-cold phosphate-buffered saline (PBS), and the final pellet was either snap frozen in liquid nitrogen for storage at −70°C or processed immediately. All subsequent procedures were carried out at 4°C. The cell pellet was resuspended in 1 volume of PBS to a total of 24 ml and sonicated on ice twice for 45 s (each) at setting 60% (Fisher sonic dismembrator), and the total cell lysate was loaded on top of six 40% (wt/vol) sucrose-PBS cushions and centrifuged in a SW-28 rotor at 25,000 rpm ($110,000 \times g$) for 2.5 h. Each of the resulting pellets was resuspended in 2 ml of 27% (wt/wt) CsCl-PBS by short-pulse sonication, pooled into two quick-seal tubes, and centrifuged to equilibrium in 27% (wt/wt) CsCl-PBS for 20 h at 28,000 rpm ($141,000 \times g$) in a SW-28 rotor. The visible band (at a density of ~1.29 g/ml) was harvested by puncturing the tubes with an 18-gauge needle, centrifuged again by using the identical conditions, dialyzed extensively against PBS, and stored at 4°C.

TEM. For transmission electron microscopy (TEM), particles were absorbed to carbon-coated grids, stained with 1% uranyl acetate, and examined with a Philips electron microscope (EM; model EM 400T) at a ×36,000 magnification as previously described (10).

Analytical gradient centrifugation of particles. To determine whether L2 was incorporated into VLP, a 12 to 45% sucrose step gradient was allowed to linearize overnight at 4°C, dialyzed samples were layered on top, and the gradient was centrifuged at 41,000 rpm ($288,000 \times g$) for 3 h in a SW-41 rotor. Fractions were harvested from the bottom and analyzed by TEM and Western blotting with monoclonal antibody Camvir-1 or the GST-16L2 rabbit antiserum. The densities of the fractions were calculated from the refractive index (20°C), as determined by an Abbe-3L refractometer (Milton Roy, Rochester, N.Y.).

Coimmunoprecipitation of L1-L2 complexes. To obtain evidence that HPV16 L1 and L2 form a stable complex, 114/K-L1 or 114/K-L1-L2 VLP preparations were immunoprecipitated in PBS (with or without 0.2% SDS) with GST-16L2 rabbit antiserum or preimmune serum and protein A-Sepharose and subjected to SDS-PAGE. Proteins were immunoblotted and probed with the anti-L1 monoclonal antibody Camvir-1 and ^{125}I-labeled anti-mouse IgG as previously described (10).

Comparison of the efficiency of VLP assembly by P/L1-L2, 114/K-L1, and 114/K-L1-L2. Three 0.5-liter cultures of insect cells were pooled, divided into three parts, and infected with P-L1/L2, K-L1, or K-L1/L2 at a similar MOI of ~10, and preparations were processed in parallel by the CsCl purification procedure described above. In initial experiments, it was determined that an MOI of ~10 resulted in maximal expression of L1 protein in insect cells. L1 protein was quantified by comparison of aliquots after SDS-PAGE and Coomassie staining to a bovine serum albumin standard. Following dialysis against PBS, VLP preparations were subjected to low-speed centrifugation ($1,100 \times g$, 5 min, Sorvall RC-3B) to remove precipitated protein. Equal volumes of VLP preparations were mixed with identical amounts of a purified herpes simplex virus (HSV) preparation (generously provided by F. Booy) and analyzed by EM at ×26,000. HSV, which is easily distinguished from HPV by size, served as an internal standard to control for differences in particle adsorption to the grid. Photomicrographs were taken from three randomly chosen fields, the number of VLP were counted and normalized for the number of HSV particles counted on the same grid, and the efficiency of assembly into VLP was estimated relative to the yield of 114/K-L1-L2 particles, which was arbitrarily set at 100%.

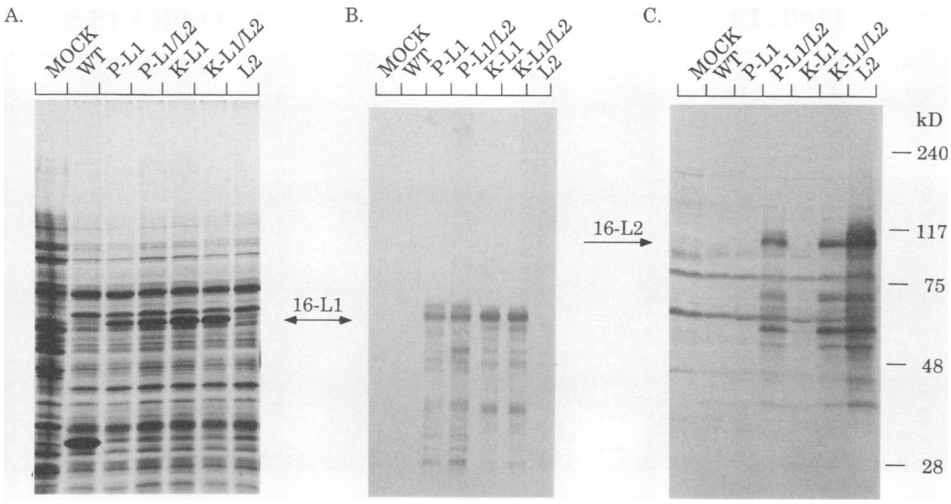

FIG. 1. Expression of HPV16 L1 and L2 proteins in insect cells via single or double recombinant baculoviruses. Insect cells were mock infected (MOCK); infected with wild-type *Autographa californica* nuclear polyhedrosis baculovirus (WT), with a single recombinant baculovirus expressing prototype L1 (P-L1), with 114/K-L1 (K-L1), or with prototype L2 (L2); or infected with double recombinant baculoviruses expressing the prototype L2 plus prototype L1 (P-L1/L2) or prototype L2 plus 114/K-L1 (K-L1/L2). After 72 h, cells were lysed by being boiled in Laemmli sample buffer, and the lysates were subjected to SDS-PAGE in 10% gels. Proteins were either stained with 0.25% Coomassie blue (A) or analyzed by Western blotting with the HPV16 L1 monoclonal antibody Camvir-1 (B) or a GST-HPV16 L2 antiserum (C) and then with ^{125}I-labeled anti-IgG.

RESULTS

Expression of HPV16 L1 and L2 capsid proteins in insect cells. We had previously found that the L1 major capsid protein from bovine PV (BPV), when expressed in insect cells infected with a single-expression recombinant baculovirus, formed VLP with high efficiency (10). The L1 protein from the prototype HPV16, however, when expressed from a similar construction, gave rise to very few particles. To compare the properties of the prototype L1 protein with those of the L1 proteins encoded by HPV16 clones isolated from condylomata acuminata, the L1 ORFs from two such HPV16 isolates, clones 114/K and 114/B, were cloned into the baculovirus single-expression vector. The prototype HPV16 L2, which encodes the minor HPV capsid protein, was also cloned into the same vector in a similar fashion. To coexpress L1 and L2 in the same cell, three double-expression vectors (L1-L2) were generated; each contained the prototype L2, expressed under the control of a synthetic promoter, plus one of the three L1 genes, expressed from the polyhedrin promoter. The synthetic promoter was chosen for expression of L2 because it is less powerful than the polyhedrin promoter; its lower expression of L2, relative to the expression of L1 from the strong polyhedrin promoter, was designed to mimic the relative abundance of L2 and L1 found in authentic virions.

Insect cells were infected with high-titer recombinant baculoviruses for 72 h, and whole-cell lysates were analyzed by SDS-PAGE and Western blotting. The respective 58-kDa L1 proteins encoded by the three L1 genes were expressed at similar levels by the single- and double-expression viruses, as demonstrated by Coomassie staining of whole-cell lysates (data for prototype [P] and 114/K [K] are shown in Fig. 1A) and their reactivities to an anti-HPV16 L1 monoclonal antibody (Fig. 1B). Differences in the patterns of the more rapidly migrating immunoreactive peptides suggested that the prototype L1 differs somewhat from the L1 proteins of the condylomata-derived strains in its susceptibility to proteolytic cleavage.

L2 protein, whether expressed from the synthetic promoter or the polyhedrin promoter, was not detected by Coomassie staining (Fig. 1A). However, Western blotting with a rabbit antiserum raised against a bacterially derived HPV16 L2 fusion protein detected a unique ~90-kDa protein in lysates from cells infected with the L2 single expressor or with the L1-L2 double-expression viruses, but not in lysates from control cells or from cells expressing L1 alone (Fig. 1C). Faster-migrating immunoreactive bands probably represent proteolytic degradation products of L2. As expected, more L2 was detected in cells infected with the single-expression vector, since L2 in this vector is under control of the polyhedrin promoter. The lack of detectable Coomassie-stained L2 protein suggests that the steady-state level of L2 protein, expressed from the same promoter, is lower than that of L1. The relative levels of L1 and L2 expressed from the L1-L2 double-expression viruses in the insect cells appears to be similar to the ratio of these two proteins in native PV virions (our unpublished results).

Efficient assembly of HPV16 L1 into virus-like particles. To determine whether the HPV16 L1 genes derived from productive lesions encode L1 proteins with the capacity to efficiently self-assemble, lysates from insect cells infected with each of the three L1 single-expression baculoviruses were subjected to cesium chloride density gradient centrifugation, and the visible band (at a density of ~1.29 g/ml) was analyzed by TEM. As previously reported (10), only rare particles were seen with preparations of the prototype L1 (data not shown). By contrast, the L1 protein from clones 114/B and 114/K self-assembled into VLP with high efficiency (Fig. 2; results shown only for 114/K-L1). The predominant structure consisted of spherical particles ~50 nm in diameter with a regular array of capsomeres, but smaller, larger, and irregular spheres as well as tubular structures were also seen. Within the resolving limits of the TEM, all the particles appeared to have the same subunit structure. There was little difference in the preparations from cells infected with the L1-L2-expressing virus compared with those infected with L1 alone. Coexpression of the prototype L2 with the condylomata-derived L1s resulted in a moderate increase in particle yield (Table 1) and did not noticeably change the size or shape distribution of the particles (Fig. 2; representative results for 114/K-L1 and 114/K-L1/L2 are shown).

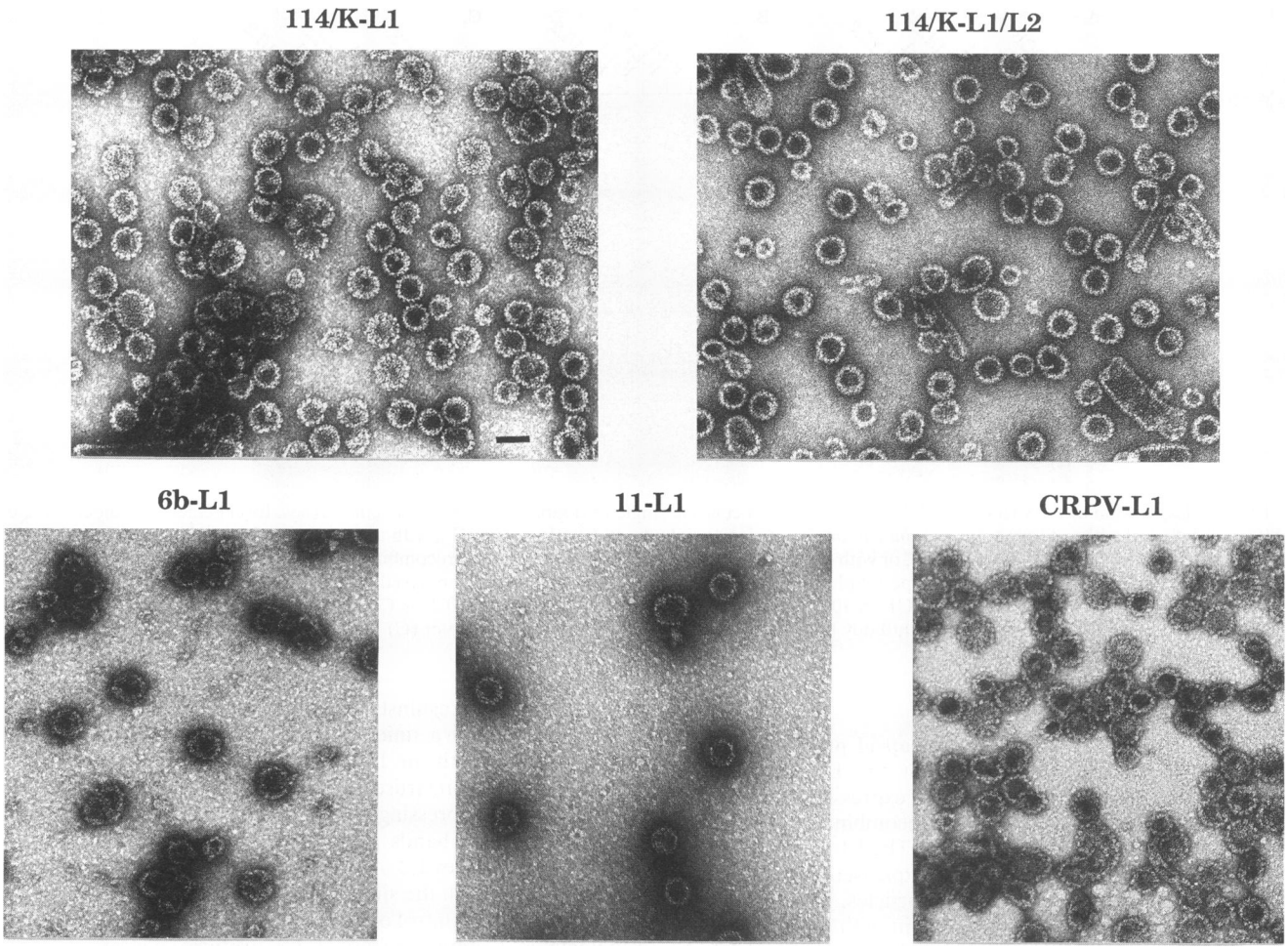

FIG. 2. Purified HPV16 L1 and HPV16 L1-L2 (clone 114/K), HPV6b L1, HPV11 L1, and CRPV L1 VLP. Particles were purified from recombinant baculovirus-infected insect cells on preparative CsCl gradients, stained with uranyl acetate, and examined by TEM. Magnification, ×36,000. Bar, 50 nm.

Cells infected with baculoviruses expressing L1 of HPV6 and HPV11 and of CRPV also contained large numbers of VLP (Fig. 2) that were morphologically indistinguishable from the HPV16 VLP. These results, together with the earlier BPV L1 and HPV11 L1 findings, indicate that self-assembly into VLP is a general feature of PV L1 and is not restricted to certain human or animal types.

Comparison of VLP yield by prototype L1-L2, 114/K-L1, or 114/K-L1-L2. To quantitate the efficiency of self-assembly into VLP, equal amounts of insect cells were infected with similar titers of recombinant baculovirus expressing prototype L1-L2, 114/K-L1, or 114/K-L1-L2 and purified in parallel as described. The total amount of L1 protein expressed in insect cells by the different baculoviruses was similar when compared by SDS-PAGE of total cell extracts (data not shown). Following purification on CsCl gradients, the amount of L1 protein recovered was comparable between the prototype L1-L2 and 114/K-L1-L2 preparations, but lower in the 114/K-L1 preparation (Table 1). However, after dialysis against PBS, most of the prototype L1 protein formed large visible precipitates and was pelleted by low-speed centrifugation. In contrast, approximately 50% of the 114/K-L1 protein was soluble after low-speed centrifugation when expressed alone or coexpressed with the prototype L2 protein (Table 1).

TABLE 1. Efficiency of HPV16 VLP assembly

Clone	L1 protein (μg)		No. of particles[a]		% Relative yield of VLP
	Total[b]	Soluble[c]	HPV	HSV[d]	
P-L1/L2	531	21	0.15*	87	0.1
K-L1	171	69	20	31	27
K-L1/L2	1,187	594	63.6	46	100

[a] Mean number of particles per field (calculated from 3 micrographs; *, 30 micrographs).
[b] Yield after CsCl purification.
[c] After low-speed centrifugation.
[d] HSV, HSV internal standard.

The relative efficiency of assembly into VLP was estimated by quantitative analysis of the different preparations by TEM. To control for differences in EM grid quality, purified HSV particles were added as an internal standard. The number of VLP on the EM micrographs was normalized for the number of HSV particles and adjusted for the volume of the VLP preparations. Results are presented as the percent relative yield of VLP compared with that of 114/K-L1-L2, which was arbitrarily set at 100%. As shown in Table 1, the yield of VLP from the 114/K-L1-L2 preparation was about 3 orders of

TABLE 2. Sequence comparison of the HPV16 L1 coding region (nt 5637 to 7154)

HPV16 isolate	Sequence[a]		
	aa 194 nt 6216	aa 202 nt 6240	aa 266 nt 6432
Prototype	gtt	cat	act
	Val	His	Thr
114/K	gtt	**G**at	act
	Val	**Asp**	Thr
114/B	**A**tt	**G**at	**G**ct
	Ile	**Asp**	**Ala**

[a] Boldface letters indicate differences from the prototype sequence. Nucleotide number refers to the nucleotides which differ from the prototype sequence. aa, amino acid.

magnitude higher than from the prototype L1-L2 preparation. In addition, coexpression of 114/K-L1-L2 resulted in about a fourfold increase of VLP yield compared with 114/K-L1 alone. These data strongly suggest that the inefficient self-assembly of prototype L1 protein into VLP is largely due to defective protein folding and/or assembly and that differences in L1 protein expression and/or degradation of L1 may contribute marginally if at all to the observed difference in VLP yield.

A single-amino-acid change prevents efficient assembly of the prototype HPV16 L1. To determine whether specific amino acid differences were responsible for the inefficient self-assembly of the prototype HPV16 L1, we sequenced the L1 ORFs of the baculovirus clones and of the original DNAs. In comparison to the prototype L1, the only difference in the entire L1 ORF of clone 114/K was a single nt 6240 C-to-G base change, which results in a nonconservative change of histidine to aspartate at amino acid 202 (Table 2). Therefore, this single-amino-acid difference must be responsible for the inefficient self-assembly of the prototype L1. As with clone 114/K, clone 114/B also encodes aspartate at amino acid 202. This latter clone contains two additional amino acid changes: Val-194 to Ile and Thr-266 to Ala (Table 2). We have also confirmed two sequencing errors in the published sequence of prototype L1 ORF (14) resulting in the insertion of CAT at nt 6900 (which encodes a highly conserved Ser) and the deletion of GAT (Asp; nt 6951 to 6953). The two condyloma-derived clones also had the corrected sequences at these sites. On the basis of these results, we consider the L1 of clone 114/K to be a wild-type HPV16 L1, clone 114/B to be a natural wild-type variant L1, and the prototype L1 to be a mutant.

Given the significant differences in the L1 genes of the three HPV16 clones, we also sequenced the L2 genes of these clones (Table 3). In addition to the previously reported sequencing error of the prototype at nt 4363 (resulting in Glu-43 encoding Asp) (20), clones 114/K and 114/B have additional nucleotide and amino acid differences. The amino acid changes, listed in Table 3, are relatively conservative, and their potential significance, if any, remains to be established.

Incorporation of L2 into VLP when coexpressed with L1 in insect cells. To determine whether L2 was incorporated into VLP, we coexpressed 114/K-L1 and the prototype L2 from a double-expression recombinant virus and purified VLP as described above. As controls, L1 and L2 were expressed alone. Following purification in two sequential CsCl gradients, the L1, L2, and L1-L2 preparations were separated on concurrently run analytical sucrose gradients, and the resulting gradient fractions were analyzed by Western blotting with antibodies specific for L1 or L2, as well as by TEM.

The density profile of the three gradients was very similar, as determined by the refractive index (data not shown). A protein with the expected ~90-kDa size was specifically recognized by the L2 antiserum in a dense gradient fraction (fraction 2) of the L1-L2 double-expression preparation but not in the preparation that expressed only L1 (Fig. 3, data shown only for the L1-L2 double-expression virus). The fraction with L2 also contained a large proportion of the immunoreactive L1 (Fig. 3), as well as many VLP by TEM (not shown). The immunoreactive protein in fractions 9 to 12 (Fig. 3, right panel) was also present in the preparation containing L1 only (data not shown), indicating nonspecific cross-reactivity of the L2 antiserum with a cellular protein. When L2 was expressed alone and purified on parallel gradients, the protein was largely degraded after the CsCl gradients and could not be detected in the fractions from the sucrose gradient (data not shown). From these results, we conclude that the prototype L2 is incorporated into VLP when coexpressed with 114/K-L1 in insect cells and that encapsidation by L1 may protect it from proteolytic degradation.

Coimmunoprecipitation of L1-L2 complexes. To obtain further evidence for complex formation between L1 and L2, we immunoprecipitated 114/K-L1 or 114/K-L1-L2 VLP preparations by using the GST-16L2 rabbit antiserum or a preimmune serum and analyzed complexes by SDS-PAGE and immunoblotting with monoclonal antibody Camvir-1 and ^{125}I-labeled anti-mouse IgG. The GST-16L2 rabbit antiserum, but not the preimmune serum, was able to precipitate a complex which contained immunoreactive L1 from VLP preparations containing L1-L2 but not from samples containing L1 alone (Fig. 4), indicating that the prototype L2 forms a complex with 114/K-L1 when coexpressed in insect cells. This complex was not disrupted when the immunoprecipitation was performed in the presence of 0.2% SDS (data not shown), indicating a stable association between the major and minor capsid proteins. The band at ~55 kDa common to all four lanes represents the

TABLE 3. Sequence comparison of the L2 coding region (nt 4235 to 5656)

HPV16 isolate	Sequence[a]					
	aa 43 nt 4363	aa 234 nt 4936	aa 301 nt 5137	aa 330 nt 5224	aa 332 nt 5230	aa 379 nt 5423
Prototype	gat	cag	att	tta	act	aat
	Asp	Gln	Ile	Leu	Thr	Asn
114/K	gat	ca**A**	at**A**	tt**T**	ac**C**	aat
	Asp	Gln	Ile	**Phe**	Thr	Asn
114/B	gat	ca**A**	att	tt**C**	act	**C**at
	Asp	Gln	Ile	**Phe**	Thr	**His**

[a] Boldface letters indicate differences from the prototype sequence. Nucleotide number refers to the nucleotides which differ from the prototype sequence. aa, amino acid.

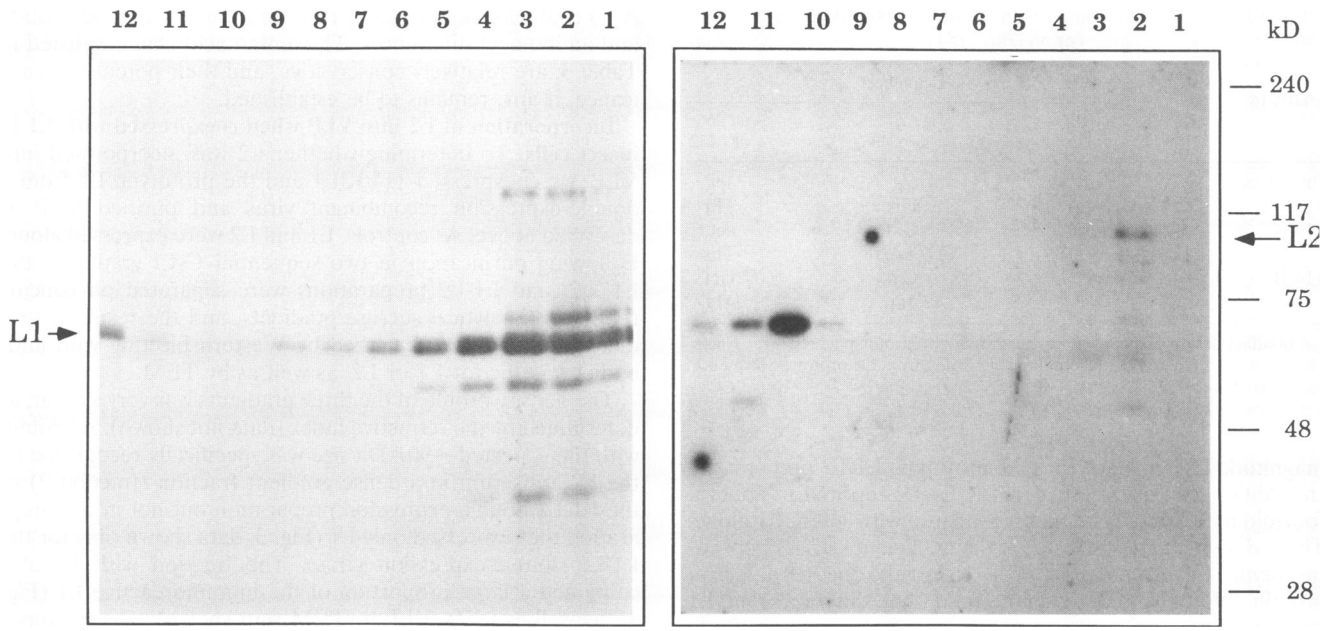

FIG. 3. Cosedimentation of HPV16 L2 with L1 when coexpressed in insect cells. Insect cells were infected with a double recombinant baculovirus expressing both HPV16 L1 (clone 114/K) and prototype L2. Following purification on CsCl gradients, the proteins were separated on a 12 to 45% sucrose gradient. Gradient fractions (numbered at the top) were analyzed by Western blotting, with HPV16 L1 monoclonal antibody Camvir-1 (left panel) or a GST-HPV16 L2 antiserum (right panel) and ^{125}I-labeled anti IgG. Fraction 1 represents the bottom and fraction 12 represents the top of the gradient.

immunoglobulin heavy chain of the primary rabbit antiserum which cross-reacts with the anti-mouse IgG secondary antibody. Similar results were obtained when Sf-9 cell extracts metabolically labeled with ^{35}S instead of purified VLP preparation were used in a similar coimmunoprecipitation experiment (data not shown).

DISCUSSION

We have identified two HPV16 L1 genes whose encoded proteins, when expressed at high levels in insect cells via a baculovirus vector, have the ability to self-assemble into VLP with high efficiency. These genes were cloned from independent HPV16 DNA isolates derived from condylomata acuminata. Their encoded L1 proteins assembled into VLP with an efficiency 3 orders of magnitude higher than that of the prototype L1, even though similar levels of L1 protein were expressed from all three L1 genes in insect cells.

Compared with the sequence of clone 114/K, L1 from the prototype strain has a single-nucleotide difference which results in a nonconserved amino acid substitution (Asp-202 to His) in the prototype L1. Thus, the difference in amino acid 202 is responsible for the inability of the prototype L1 to self-assemble efficiently. Amino acid sequence alignment of L1 ORFs of human and animal PV indicates that aspartic acid or glutamic acid is present in all of them (data not shown), and the various L1 proteins we have found to self-assemble efficiently each encode one of these two amino acids at this position. We therefore consider the L1 of clone 114/K to be a wild-type gene and the prototype L1 a mutant. In addition, clone 114/B, which has two other changes, is likely to be a natural wild-type variant, since it assembles as efficiently as clone 114/K. It remains to be determined whether the functional alteration in L1 of the prototype strain was a random

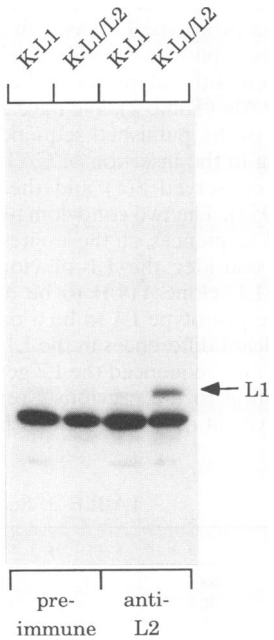

FIG. 4. Coimmunoprecipitation of HPV16 L1 with L2 when coexpressed in insect cells. Purified VLP obtained from insect cells infected with a recombinant baculovirus expressing both HPV16 L1 (clone 114/K) and prototype L2 (K-L1/L2) or HPV16 L1 alone (K-L1) were immunoprecipitated with preimmune serum or GST-HPV16 L2 antiserum, subjected to SDS-PAGE, and analyzed by Western blotting with HPV16 L1 monoclonal antibody Camvir-1 and ^{125}I-labeled anti-mouse IgG.

event or whether the mutation might have a role in oncogenic progression.

Our results indicate that the L1 of a high-risk mucosal HPV type has the same intrinsic ability to form VLP as L1 of a cutaneous type (7, 10), when expressed in insect cells and that L2 or other viral proteins are not required for efficient assembly. We have similarly expressed the L1s of HPV6b, HPV11, and CRPV and have obtained efficient self-assembly with each of these proteins. Our results for HPV11 differ somewhat from that of Rose et al. (16), who obtained mainly aggregates and rather inefficient VLP formation after extraction of HPV11 L1 from insect cells, although VLP were readily observed in electron micrographs of thin sections of infected insect cells. Differences in extraction and purification procedures or the use of a different HPV11 isolate may account for the different efficiency of VLP isolation.

The precise location of L2 within the PV virion and its biological function are unknown. We have observed only about a fourfold increase in the yield of VLP by coexpressing L1-L2 of HPV16 compared with L1 alone, although our evidence strongly suggests that L2 was incorporated into the VLP. Similar results were obtained by coexpressing L1 and L2 of BPV1 or CRPV (data not shown). In contrast, Hagensee et al. reported a 10- to 100-fold increase in particle yield when L1-L2 of HPV1, as opposed to L1 alone, were expressed via recombinant vaccinia viruses (7), although the method used for quantitation was not reported. These divergent results might be explained by differences in the expression systems or by more fundamental differences between the L1-L2 proteins of cutaneous and genital HPV. We favor the former possibility. The baculovirus expression system generally produces more recombinant protein than does the vaccinia virus system, and the L1 proteins of BPV1 and CRPV self-assemble efficiently when overexpressed, although they are cutaneous viruses. It remains possible that the prototype HPV16 L2 might be defective, but it seems unlikely that this would also be true of BPV L2 and CRPV L2, since the latter clones were isolated from infectious virion preparations. In preliminary experiments, we have coexpressed 114/K-L1 and 114/K-L2 and have obtained similar amounts of VLP compared with 114/K-L1 coexpressed with the prototype L2 (unpublished observation).

The ability to generate preparative amounts of HPV16 VLP may be critical for the development of a sensitive serological assay to measure HPV16 virion antibodies in humans. The results of serological tests using bacterially derived proteins or synthetic peptides have not correlated well with other measures of HPV infection (reviewed in reference 4). However, significant antivirion immune responses might have escaped detection, since the majority of epitopes recognized after experimental inoculation with animal PV virions are conformationally dependent (5, 10). We have recently tested sera of women with known HPV status for reactivity in an HPV16 enzyme-linked immunosorbent assay (ELISA) using 114/B-VLP as the antigen (10a). The sera of a majority of women whose cervicovaginal specimens were positive for HPV16 DNA reacted positively in the ELISA, whereas the sera of most women negative for HPV DNA or positive for low-risk HPV6 or HPV11 DNA were negative in the ELISA. In contrast, when the ELISA used the L1 protein from the prototype HPV16, the results were completely negative for the sera of women who were HPV16 DNA positive.

Efficient self-assembly of HPV16 particles may also have implications for efforts to develop an effective prophylactic vaccine against HPV16 infection. In previous vaccination experiments, bacterially expressed capsid proteins were partially effective in preventing experimental infection in animals, although they induced only low levels of neutralizing antibodies (9, 11, 15). We recently reported that immunization of rabbits with native, but not denatured, VLP from BPV L1 induced high-titer neutralizing antibodies (10). This observation suggests that VLP might have the potential to induce long-lasting immunity, a characteristic that might be advantageous for an effective HPV vaccine.

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